Purification and Properties of 3-Deoxy-D-Arabinoheptulosonic Acid-7-Phosphate Synthetase (trp) from *Escherichia coli*

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The 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthetase which is subject to regulation by tryptophan has been partially purified from a strain of *Escherichia coli* K-12, in which this is the only functional form of this enzyme activity, and from a similar strain possessing a feedback-resistant form of the enzyme. Maximal observed inhibition by tryptophan of the feedback-sensitive enzyme was 56%. There was no evidence for cooperativity in the saturation of the enzyme with tryptophan or E4P. The molecular weights of the feedback-sensitive and feedback-resistant forms of the enzyme were the same (52,000), and no change was detected in the molecular weight of the feedback-sensitve enzyme in the presence of tryptophan. The effect of tryptophan analogues was tested to determine the nature of the tryptophan binding site. Treatment with ethylenediaminetetraacetic acid removed 80% of the activity of the feedback-sensitive enzyme. This activity was restored upon the addition of Co^{2+} or Mn^{2+}. Neither treatment with ethylenediaminetetraacetic acid nor addition of Co^{2+} or Mn^{2+} affected the activity of the feedback-resistant enzyme.

The first reaction of aromatic biosynthesis is the condensation of phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate (E4P) to form 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP), and is carried out in *Escherichia coli* K-12 by three isofunctional DAHP synthetase enzymes (EC 4.1.2.15) (4, 21, 24, 25). The activity of DAHP synthetase (phe) is inhibited by phenylalanine, and the activity of DAHP synthetase (tyr) is inhibited by tyrosine. Early reports (4, 14, 24) suggested that DAHP synthetase (trp) was not inhibitable by tryptophan. However, Doy (10) reported a 32% inhibition of DAHP synthetase (trp) in crude extracts of *E. coli* W, and Pittard et al. (19) conclusively demonstrated inhibition of this enzyme in *E. coli* K-12 by both in vivo and in vitro studies using crude extracts. The maximal inhibition observed in vitro using crude extracts was 60% and was obtained with 1 mM tryptophan in the presence of 1 mM Co^{2+}. To confirm and extend these studies DAHP synthetase (trp) has been partially purified from *E. coli* K-12, and its properties, particularly those relating to feedback inhibition, have been studied. In addition, a feedback-resistant form of this enzyme has also been partially purified and its properties were compared to those of the

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feedback-sensitive enzyme. A preliminary account of this work was recently reported (5).

MATERIALS AND METHODS

Organisms. The strains used in this study were JP2192 and JP68. Strain JP2192 (*aroF363, aroG365, aroJ385, trpR363, thi-1*) is a mutant strain derepressed for the synthesis of feedback-sensitive DAHP synthetase (trp), and lacks functional enzymes for DAHP synthetases (tyr) and (phe). Strain JP68 (*aroF363, aroG365, aroH381, trpR363, thi-1, his-4*) is a mutant strain derepressed for the synthesis of feedback-resistant DAHP synthetase (trp), and lacks functional enzymes for DAHP synthetases (tyr) and (phe). This strain has been described previously (19). The genetic symbols have been described in previous publications (6, 19).

Chemicals. Sephadex G-25, Sephadex G-100, and diethylaminoethyl-Sephadex were obrained from Pharmacia Fine Chemicals. Hydroxylapatite was obtained from Bio-Rad Laboratories, Richmond, Calif.

All tryptophan analogues were rigorously tested for purity (in particular, to check for contamination with tryptophan) by D. Rivett, CSIRO Division of Protein Chemistry, Parkville, Australia. The tests used were thin-layer chromatography on cellulose and Silica Gel G in methyl ethyl ketone-acetic acid-water (4:1:1); high-voltage electrophoresis (pH 1.9, 3 kV, 60 V/cm); analysis using the amino acid analyzer by the method of Nicholls and Rivett (18). All analogues were found to be pure except for 1-methyl-DL-tryptophan which contained about 1% free tryptophan.

D-Erythrose-4-phosphate dimethylacetal dicyclo-hexyl-ammonium salt was obtained from Fine Chemicals of Australia and was converted to free D-erythrose-4-phosphate by the method of Ballou et al. (3). Other chemicals were generally of the highest quality obtainable commercially, and unless specified were not further purified.

Polyacrylamide gel electrophoresis. The system for gel electrophoresis was that described by Davis (8). The concentration of acrylamide in the separating gel was 7%. Gels were stained with Coomassie blue.

Protein estimations. Protein was estimated by the method of Lowry et al. (15), with bovine serum albumin (BSA) as a standard.

In the case of column fractions, the optical density at 280 nm was read, assuming this to be directly proportional to the protein concentration.

Assay of DAHP synthetase. DAHP synthetase activity was determined by measuring the amount of DAHP formed from E4P and PEP, based on the method of Srinivasan and Sprinson (22). In all assays with partially purified enzyme, unless otherwise stated, the incubation mixture contained 0.5 µmol of PEP, 0.58 µmol of E4P, and a rate-limiting amount of enzyme, in 0.005 M phosphate buffer (pH 6.4 or 7) containing 0.1% BSA. The total volume of the assay mixture was made up to 0.5 ml with distilled water. The reaction was initiated by the addition of enzyme, and after 10 min of incubation at 37 C it was stopped by the addition of 0.1 ml of 10% trichloroacetic acid. The mixture was centrifuged and 0.4 ml of supernatant was removed for assay of DAHP. DAHP was estimated by the method of Doy and Brown (12). In this assay DAHP is oxidized to yield a compound that reacts with thiobarbituric acid to give a chromophore with an absorption maximum at 549 nm. The concentration of DAHP was calculated by using the molar extinction coefficient of 68,000 (13).

When inhibition was tested by inclusion of tryptophan or tryptophan analogues in the assay, the procedure was modified to account for any interference by these inhibitors with the assay for DAHP. In such cases, for each concentration of tryptophan or tryptophan analogue, four assays were carried out. In two of these the inhibitor was present during the enzyme assay, and in the other two the inhibitor was added after the trichloroacetic acid. Dowex-50 ion-exchange resin was then added (Dowex-1 was used in the case of analogues where the α-amino group was either substituted or absent), the solution was mixed by Vortexing for half a minute, and the resin and protein precipitate were removed by centrifugation. Four-tenths milliliter of the supernatant was assayed for DAHP. The percentage of inhibition was calculated by comparing the average value for enzyme assays carried out in the presence of inhibitor with the average value for assays in the absence of inhibitor.

In all kinetics experiments, only values which represent less than 15% substrate utilization were accepted for use in calculation of initial velocites.

Unit of enzyme activity. One unit of enzyme activity was taken as that amount of enzyme which

produced 0.1 µmol of product in 20 min at 37 C. In plots of initial velocity versus substrate concentration, the initial velocity is expressed as the micromoles of DAHP formed per minute per milligram of protein. Specific activities of enzyme preparation were expressed as the units of enzyme activity per milligram of protein.

Computer analysis of kinetic data. K_m and V_{max} values were obtained by fitting the kinetic data directly to the equation $v = V_{max}S/K_m + S$ where v is the initial velocity, V_{max} is the maximal initial velocity, K_m is the Michaelis constant, and S is the variable substrate concentration. This method avoids errors associated with obtaining the constants from double-reciprocal plots. It involved the use of a computer program for the least squares estimation of nonlinear parameters. This program was kindly provided by B. Davidson, Department of Biochemistry, Melbourne University. An IBM 7044 computer was used. Standard errors were calculated at the 95% level.

Growth of cells. For the large-scale purification of DAHP synthetase (trp), 300-liter batch cultures were grown at Commonwealth Serum Laboratories, Parkville, Australia. The minimal medium used was medium 56, described by Monod et al. (17), supplemented with 0.5% glucose as the carbon source, thiamine, and amino acid requirements. The cells were collected by centrifugation, and the cell paste was transferred into plastic bags and frozen in the form of thin sheets.

Purification of DAHP synthetase (trp). Preliminary experiments with crude extracts suggested that DAHP synthetase (trp) is most stable in the presence of 0.5 mM Co^{2+} and 0.5 mM PEP. Therefore, all buffers used in the purification contained 0.5 mM Co^{2+} and 0.5 mM PEP. All operations were conducted at 4 C. Both forms of the enzyme were purified using similar steps.

Step 2: preparation of crude extracts. Frozen cells were thawed slowly (4 C) in 0.9% NaCl. After uniform suspension, the cells were harvested by centrifugation and washed once more in 0.9% NaCl. The cells were harvested again by centrifugation and suspended in 0.05 M phosphate buffer, pH 7, containing 0.5 mM PEP and 0.5 mM Co^{2+} (4 ml/g [wet weight]). The suspension was homogenized and passed through cheese cloth to remove mucoid clumps. The cells were disrupted at 20,000 lb/in² using a Ribi Cell fractionator. Crude extracts were obtained by centrifugation at 21,000 $\times$ g to remove cell debris.

Step 2: precipitation of nucleic acids. Two percent protamine sulfate was added dropwise to the crude extract over a period of 10 min, while maintaining constant stirring (1 ml of 2% protamine sulfate/140 mg of protein in the crude extract). Stirring was continued for a further 30 min, and the precipitate was removed by centrifugation at 18,000 $\times$ g for 20 min.

Step 3: ammonium sulfate fractionation. Finely ground ammonium sulfate powder was slowly added to the supernatant from step 2 until 40% saturation

was reached (9). The suspension was stirred for a further 30 min, and the precipitate was collected by centrifugation at 18,000 × g for 15 min. The supernatant was removed and more ammonium sulfate was added till 55% saturation was reached. The suspension was stirred for a further 30 min and the second precipitate was collected as above. This was dissolved in 0.01 M phosphate buffer, pH 7, containing 0.5 mM PEP and 0.5 mM Co^{2+} (buffer A), and dialyzed against two changes of the same buffer.

Step 4: chromatography on diethylaminoethyl-Sephadex. The dialysate from step 3 was adjusted to 0.07 M KCl and applied to a column (4.6 by 30 cm) of diethylaminoethyl-Sephadex that had been previously equilibrated in buffer A containing 0.07 M KCl. The flow rate was 60 ml/h and 15-ml fractions were collected. After the column was washed with approximately 500 ml of the same buffer, a 2,000-ml linear gradient in KCl concentration was applied (0.07 to 0.40 M KCl in buffer A). A single peak of DAHP synthetase activity was eluted and fractions containing this activity were pooled and then concentrated by ultrafiltration with a Diaflo PM-10 membrane. The concentrate was dialyzed against buffer A.

Step 5: chromatography on hydroxylapatite. The dialysate from step 4 was adjusted to 0.05 M in phosphate concentration and applied to a column (20 by 2.7 cm) of hydroxylapatite that had been previously equilibrated in 0.05 M phosphate buffer, pH 7, containing 0.5 mM PEP and 0.5 mM Co^{2+}. The flow rate was 60 ml/h. After the column was washed with approximately 200 ml of the same buffer, a 1,000-ml linear gradient in phosphate concentration was applied (0.05 to 0.30 M phosphate, pH 7, containing 0.5 mM Co^{2+} and 0.5 mM PEP). A single peak of DAHP synthetase activity was eluted; fractions containing this activity were pooled and concentrated by ultrafiltration using a Diaflo PM-10 membrane; and the concentrate was dialyzed against buffer A.

Determination of apparent molecular weight by gel filtration on Sephadex G100. The method used for determination of apparent molecular weight was based on that of Andrews (1). BSA, ovalbumin, and cytochrome c were used as standards, whereas the void volume was estimated by using blue dextran.

RESULTS

Purification of DAHP synthetase (trp). The enzyme was purified as described.

A summary of the purification procedure is presented in Table 1. The material after the hydroxylapatite step possessed a specific activity of DAHP synthetase (trp) which was about 216-fold higher than that of the starting material in the case of the feedback-sensitive enzyme, and 152-fold higher in the case of the feedback-resistant enzyme. This represents an increase in specific activity over that present in crude extracts of wild-type cells of 670 and 471, respectively. It can be seen from Table 1 that the yield of the feedback-resistant enzyme was

significantly lower than that of the feedback-sensitive enzyme. The increased instability of this enzyme was apparent during steps 3, 4, and 5. No further purification beyond the hydroxylapatite step was attempted. All subsequent work described in this paper utilizes the enzyme preparation from the hydroxylapatite step. Polyacrylamide gel electrophoresis of the partially purified material showed that it contained at least three major bands in both cases. Attempts to demonstrate the location of DAHP synthetase activity (by elution of protein from slices of unstained gels) were unsuccessful. This was presumably due to lability of the enzyme under the conditions used.

Estimation of molecular weight of DAHP synthetase (trp) by gel filtration. Gel filtration on Sephadex G-100 was carried out as described. A sample (approximately 500 μg) of the enzyme was mixed with standards and blue dextran, and applied to a Sephadex G-100 column previously equilibrated in 0.1 M phosphate buffer (pH 7) containing 0.5 mM Co^{2+} and 0.5 mM PEP. DAHP synthetase (trp) activity in both cases was eluted as a single symmetrical peak. The ratio of elution to void volume, corresponding to DAHP synthetase (trp) activity, indicated an apparent molecular weight of 52,000 for both the feedback-sensitive and feedback-resistant forms of the enzyme. The data for the feedback-sensitive enzyme are shown in Fig. 1. Addition of 0.5 mM L-tryptophan to the buffer failed to alter the elution behavior of the feedback-sensitive enzyme. Therefore on this criterion, no aggregation or dissociation of this enzyme occurs in the presence of the allosteric ligand tryptophan.

Substrate saturation kinetics using feedback-sensitive enzyme: (i) E4P as variable substrate. Initial velocity was measured at various concentrations of E4P in the presence of 1 mM Co^{2+} and a constant saturating concentration of PEP. Initial velocity versus initial substrate plots were linear (Fig. 2), indicating that normal Michaelis-Menten kinetics are followed. The apparent K_m of the enzyme for E4P is 0.076 ± 0.005 mM, and the apparent V_{max} is 4.31 ± 0.09 μmol per min per mg of protein. Substrate saturation kinetics for E4P were also carried out in the presence of 50 μM tryptophan. The double-reciprocal plot (Fig. 2) is again linear. As both the slope and the intercept are altered in the presence of tryptophan, the inhibition by tryptophan is most probably of the noncompetitive type (7) with respect to E4P. Computer analysis also revealed that the best fit of the data was to the general noncompetitive

TABLE 1. *Purification of DAHP synthetase (trp)*

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Fold purification
1. Crude extract	2,200	73,000	580,000	8 (5.2)[a]	100 (100)	1 (1)
2. Protamine sulfate	2,540	53,000	575,000	10.8 (8.7)	99 (99.5)	1.3 (1.7)
3. Ammonium sulfate (40%–55% saturation)	340	11,600	322,000	27.8 (15.8)	55 (42)	3.5 (3)
4. Diethylaminoethyl-Sephadex	85	1,060	255,000	240 (81)	44 (17)	30 (16)
5. Hydroxylapatite	14	82	142,000	1,730 (790)	25 (5.2)	216 (152)

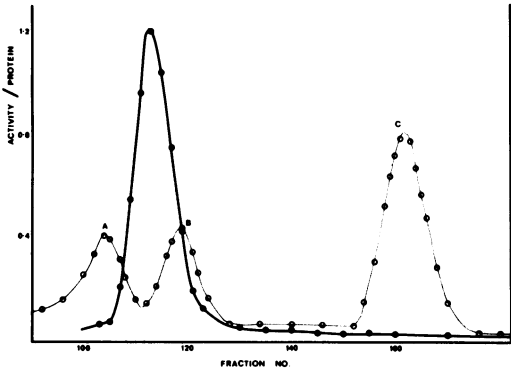[a] Values in parenthesis are for the feedback-resistant enzyme.

FIG. 1. *Elution profile of partially purified DAHP synthetase (trp) (feedback sensitive), together with standards, on a column of Sephadex G-100 previously equilibrated in 0.1 M phosphate buffer, pH 7, containing 0.5 mM PEP and 0.5 mM Co²⁺. Blue dextran was eluted at an elution volume of 158 ml (Vₒ). (A) BSA; (B) ovalbumin; (C) cytochrome c. Symbols: ●, DAHP synthetase activity. (optical density 549 nm per 50 μliters of column fraction); ○, protein. (optical density 280 nm; optical density 412 nm in the case of cytochrome c).*
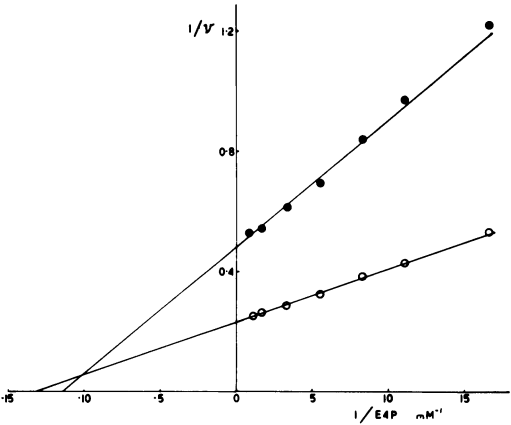

FIG. 2. *Double-reciprocal plots of the effect of E4P concentration on the initial velocity (v) in the presence of 1 mM Co²⁺ (○), and in the presence of 1 mM Co²⁺ and 0.05 mM tryptophan (●). PEP (1 mM) was present in both cases, in 0.005 M phosphate buffer (pH 6.4) containing 0.1% BSA. The lines shown are those predicted by the computer (as described in Materials and Methods) while the experimentally derived values are indicated.*

inhibition equation of Cleland (7). From a Hill plot (2) of the above data, the Hill coefficient was found to be 1, both in the presence and absence of tryptophan, indicating the absence of cooperativity between E4P binding sites.

Kinetic studies were also carried out on the feedback-sensitive enzyme in the absence of any added Co²⁺, and in this case the V_{max} was found to be 3.75 ± 0.08 μmol per min per mg of protein. This is a significant reduction from the value of 4.31 ± 0.09 μmol per min per mg of protein obtained in the presence of added (1 mM) Co²⁺.

(ii) **PEP as variable substrate.** A sample of the partially purified feedback-sensitive enzyme preparation was passed through a Sephadex G-25 column equilibrated with 0.01 M phosphate buffer, pH 7, to remove PEP from

the preparation. It was found that the initial velocity did not vary with PEP concentration over the range 0.06 mM PEP to 0.90 mM PEP, indicating that PEP is saturating over this concentration range. The DAHP assay used is not sensitive enough to permit initial velocity determinations at lower PEP concentrations.

Inhibition by L-tryptophan. Inhibition of DAHP synthetase (trp) activity (feedback-sensitive enzyme) by various concentrations of tryptophan was tested, and the results in the presence and absence of 1 mM Co²⁺ are shown in Fig. 3. No sigmoidality is evident in this curve. The maximal inhibition observed was 56% in the presence of 1 mM Co²⁺ and 46% in the absence of added Co²⁺.

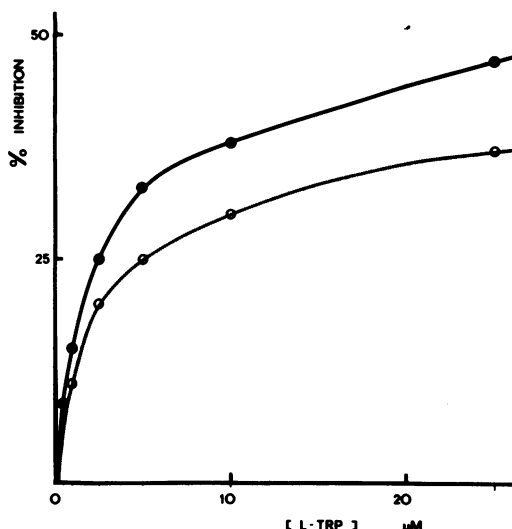As inhibition is not 100% at saturating concentrations of tryptophan, a modified Hill plot

FIG. 3. *Percentage inhibition of DAHP synthetase (trp) (feedback sensitive) by varying concentrations of tryptophan. All assays contained 1.17 mM E4P, 1 mM PEP, and 1.5 μg of protein from the hydroxylapatite fraction. Symbols:* ●, *1 mM Co²⁺ added to assays;* ○, *no added Co²⁺.*

(2) was used to test for cooperativity between tryptophan binding sites. The slope was found to be approximately 0.9 for inhibition both in the presence and in the absence of Co^{2+}, indicating the absence of cooperativity between tryptophan binding sites. Feedback-resistant enzyme from strain JP68 was found to be completely insensitive to tryptophan in the range 0.5 μM tryptophan to 1 mM tryptophan, whether in the presence or absence of 1 mM Co^{2+}.

Effect of analogues of L-tryptophan on the activity of the feedback-sensitive enzyme. A number of analogues of tryptophan were tested for their ability to inhibit DAHP synthetase (trp) (Table 2). The following can be concluded from these reults. (i) Only the L-isomer of tryptophan causes inhibition. (ii) The carboxyl group is absolutely essential for inhibition. This is apparent from the fact that tryptamine (which lacks the carboxyl group) does not cause any inhibition. In cases where the carboxyl group is blocked by substitution (L-tryptophan methyl ester, L-tryptophanamide), a significant decrease in inhibition occurs. (iii) The α-amino group is not absolutely essential for inhibition, since indole propionic acid and indole lactic acid are capable of causing significant inhibition. The reduced ability of N-acetyl-DL-tryptophan to inhibit may be due to steric hindrance by the bulky acetyl group. (iv) Presence of the β

TABLE 2. *Inhibition of DAHP synthetase (trp) by analogues of tryptophan and other related molecules*

Inhibitor[a]	% Inhibition[b]
L-Tryptophan	55
D-Tryptophan	0
L-Tryptophan methylester	17
L-Tryptophan amide	15
Tryptamine	0
3-Indole lactic acid	33
3-Idole propionic acid	37
3-Indole acetic acid	0
Indole propionyl glycine	0
(3-Indolyl) mercaptoacetic acid	34
N-acetyl-DL-tryptophan	14
1-Methyl-DL-tryptophan	14
2-Methyl-DL-tryptophan	50
4-Methyl-DL-tryptophan	45
5-Methyl-DL-tryptophan	30
6-Methyl-DL-tryptophan	15
7-Methyl-DL-tryptophan	17
7-Aza-tryptophan	14
Kynurenine	9
o-Nitrophenacyl glycine	0
Histidine[c]	0
Phenylalanine[c]	0
Tyrosine[c]	0
Chorismic acid[c]	0
p-Amino benzoic acid[c]	0
p-Hydroxy benzoic acid	0
2,3-Dihydroxybenzoic acid[c]	0

[a] All inhibitors were tested at 1 mM concentrations of the L equivalent in the presence of 1 mM Co^{2+}, 1 mM PEP, 1.17 mM E4P and 1.5 μg of protein from the hydroxylapatite fraction.

[b] These inhibitors were also tested at 0.2 mM concentrations in the presence of 1 mM trp, 1 mM Co^{2+}, 1 mM PEP, 1.17 mM E4P and 1.5 μg of protein from the hydroxylapatite fraction, and in no case was the inhibition different from that given by 1 mM tryptophan alone.

[c] Percent inhibition was calculated using appropriate controls to take into account any effect of the inhibitor on the assay for the estimation of DAHP.

carbon is essential for inhibition, although its role is most likely that of a spacer group. This is indicated by the failure of indole acetic acid to inhibit, while (3 indolyl) mercaptoacetic acid (β carbon replaced by sulfur) inhibits to the same extent as indole propionic acid. (v) The indole nitrogen is important in inhibition as is apparent from the low inhibition by 1-*methyl* tryptophan. (vi) The pyrrole ring of the indole nucleus is required for maximal inhibition. This is demonstrated by the inability of kynurenine (o-amino phenyacyl glycine) to significantly inhibit enzymic activity, and the failure of o-nitrophenacyl glycine to cause any inhibition. (vii) Methyl substitutions in the 6 and 7 posi-

tions of the indole ring considerably affect the capacity to inhibit.

Phenylalanine, tyrosine, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, chorismic acid, and histidine do not inhibit DAHP synthetase (trp), nor do they increase the maximal inhibition given by tryptophan (Table 2). The latter result appears to rule out the possibility of cumulative type inhibition by tryptophan and one of these compounds.

Effect of EDTA and divalent cations: (i) feedback-sensitive enzyme. After 20 min of preincubation of enzyme with 0.1 mM ethylenediaminetetraacetic acid (EDTA), 80% of activity was lost (Table 3). Co^{2+} and Mn^{2+} could restore this activity (Co^{2+} causing an activation), and Ni^{2+} and Ca^{2+} could partially restore this activity (Table 3). When 1 mMCo^{2+} was present at the same time as EDTA, no activity was lost. In the presence of 1 mM PEP during the 20 min of incubation with EDTA, only 40% of the activity was lost.

(ii) Feedback-resistant enzyme. Neither 0.1

TABLE 3. *Effect of EDTA[a] and divalent metal cations[b] on DAHP synthetase (trp) activity[c] (feedback-sensitive enzyme)*

Addition		Activity remaining (%)
Preincubation	Enzyme assay	
No preincubation		100
None	None	100
EDTA	None	20
EDTA	Zn^{2+}	16
EDTA	Mn^{2+}	100
EDTA	Co^{2+}	118
EDTA	Mg^{2+}	32
EDTA	Ni^{2+}	75
EDTA	Fe^{2+}	10
EDTA	Cu^{2+}	20
EDTA	Ca^{2+}	65
1 mM PEP	None	100
EDTA + 1 mM PEP	None	60
EDTA + Co^{2+}	None	115
No preincubation	EDTA	60
No preincubation	EDTA + Co^{2+}	112
No preincubation	EDTA + Mn^{2+}	70

[a] Ethylenediaminetetraacetic acid. This was used at 0.1 mM.

[b] Divalent metal cations were added at1 mM.

[c] Enzyme (1.5 µg of protein from the hydroxylapatite fraction) was preincubated at 0 C for 20 min in 0.005 M phosphate buffer, pH 7, containing 0.1% BSA, and in this buffer with various additions. After 20 min, additions were made to the mixture and the enzyme reaction was initiated by addition of 1 mM PEP and 1.17 mM E4P.

mM EDTA nor 0.5 mM EDTA had any effect on the activity of the feedback-resistant enzyme when tested under the same conditions as above. Also 1 mM Co^{2+} or 1 mM Mn^{2+} did not stimulate the activity of this enzyme.

Effect of pCMB and mercaptoethanol. Thirty micrograms of protein from the hydroxylapatite fraction was incubated for 20 min at room tempreature (25 C) in 0.01 M phosphate buffer containing 1 mg of BSA per ml and 0.01 mM *p*-chloromercuribenzoate (pCMB). Samples of the treated enzyme were then removed into a normal assay mixture, such that a 1:100 dilution was effected, and DAHP synthetase (trp) activity was assayed. Under these conditions the feedback-sensitive enzyme lost 78% of its activity and the feedback-resistant enzyme lost 50% of its activity relative to controls containing no added pCMB. The substrate PEP completely protected both forms of the enzyme against pCMB inactivation. Therefore, it seems likely that pCMB is reacting with sulfhydryl groups in the PEP-binding site(s) or catalytic site(s) of the enzyme. Mercaptoethanol (1mM) was found to have no effect on the activity of either form of the enzyme.

DISCUSSION

Molecular weight studies. The finding that the apparent molecular weight of the feedback-sensitive enzyme was unaffected by tryptophan is in contrast to the situation in *Neurospora crassa* where DAHP synthetases (phe) and (tyr) dissociate to half molecules in the presence of phenylalanine and tyrosine, respectively (11). DAHP synthetase (phe) from *E. coli* K-12 also fails to show a change in molecular weight in the presence of its allosteric effector, phenylalanine (21).

A study of the molecular weight of the feedback-resistant enzyme indicates that the particular alteration which results in feedback resistance in this enzyme has not caused a detectable decrease in molecular weight. This fact does not exclude the possibility that DAHP synthetase (trp) normally possesses a separate regulatory subunit which binds only tryptophan, though there is no evidence for such a subunit from any of the work described.

Role of cobalt. It appears likely that Co^{2+} or some other divalent cation, e.g., Mn^{2+}, is required for normal activity and inhibition of the feedback-sensitive form of DAHP synthetase (trp). The different levels of inhibition observed in the absence of added Co^{2+}, in crude cell extracts (19) compared to partially purified

material, probably reflect differences in the amount of Co^{2+} already bound to the enzyme. In the latter case enzyme purification had been carried out in buffers containing 0.05 mM Co^{2+} and the enzyme may be partially saturated with Co^{2+} even after a 4,000-fold dilution in buffer. The observation that PEP partially protected the feedback-sensitive enzyme from inactivation by EDTA could indicate that in the presence of PEP, Co^{2+} is either more tightly bound to the enzyme or less accessible to the action of EDTA. This could result from conformational changes induced by PEP or from direct protection by the bound PEP molecules.

In contrast to these results, the feedback-resistant enzyme is not inactivated by treatment with EDTA nor is it possible to demonstrate inhibition by tryptophan in the presence of added Co^{2+}. This result could be explained if a conformational change had occurred in the feedback-resistant enzyme such that Co^{2+} was now more tightly bound than in the sensitive enzyme and could no longer be removed by EDTA, or alternatively if the change in structure of the feedback-resistant enzyme rendered the role of Co^{2+} redundant for normal catalytic activity. In both cases the changes affecting sensitivity to EDTA must also affect inhibition of enzyme activity by tryptophan. To distinguish between these two possibilities it will be necessary to examine pure preparations of both the feedback-sensitive and feedback-resistant enzymes.

Nature of tryptophan inhibition and of the tryptophan-binding site. Evidence from Hill plots of inhibition data suggests that no cooperativity exists between tryptophan-binding sites, nor does tryptophan promote cooperativity between E4P-binding sites. Tryptophan appears to exhibit a noncompetitive type of inhibition with respect to E4P. From studies with tryptophan analogues it is possible to consider the types of interactions which may be occurring between tryptophan and its binding site on synthetase (trp). It should be pointed out that from these studies with analogues it is difficult to state which interactions are necessary for binding and which interactions are required to induce the appropriate conformational changes. The negatively charged α-carboxyl group of tryptophan is essential for inhibition and probably forms an electrostatic bond with a positively charged group on the protein. The distance of this carboxyl group from the indole ring is very important, as shortening the side chain abolishes inhibition. Another point where interaction probably occurs with the protein is

through the indole nitrogen, as a methyl substitution here severely reduces capacity to inhibit. The indole-NH could hydrogen bond with a group on the protein. The pyrrole ring of the indole nucleus is important, presumably because it can maintain the inhibitor in an appropriate conformation for fitting into the binding site, e.g., maintaining relative positions of α-carboxyl group and indole-NH. As bulky substitutions in the 6 and 7 positions of the indole ring considerably diminish ability to inhibit, it appears likely that residues of the protein (probably nonpolar) are in close proximity to tryptophan in this region.

LITERATURE CITED

1. Andrews, P. 1965. The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
2. Atkinson, D. E. 1966. Regulation of enzymic activity. Ann. Rev. Biochem. **35**:85–124.
3. Ballou, C. E., H. O. L. Fischer, and D. L. MacDonald. 1955. The synthesis and properties of D-erythrose-4-phosphate. J. Amer. Chem. Soc. **77**:5967–5970.
4. Brown, K. D., and C. H. Doy. 1963. End product regulation of the general aromatic pathway in *Escherichia coli* W. Biochim. Biophys. Acta **77**:170–172.
5. Camakaris, J. 1973. Purification and properties of DAHP synthetase (trp) from *E. coli* K-12. Proc. Aust. Biochem. Soc. **6**:2.
6. Camakaris, J., and J. Pittard. 1971. Repression of 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthetase (trp) and enzymes of the tryptophan pathway in *Escherichia coli* K-12. J. Bacteriol. **107**:406–414.
7. Cleland, W. W. 1963. The kinetics of enzyme-catalysed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. Biochim. Biophys. Acta **67**:173–187.
8. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to serum proteins. Ann. N. Y. Acad. Sci. **121**:404–427.
9. Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones. 1969. Data for biochemical research, 2nd ed. Clarendon Press, Oxford.
10. Doy, C. H. 1967. Tryptophan as an inhibitor of 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthetase. Biochem. Biophys. Res. Commun. **26**:187–192.
11. Doy, C. H. 1970. Isoenzymes and allosteric inhibition of *Neurospora crassa* 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase. Biochim. Biophys. Acta **198**:364–375.
12. Doy, C. H., and K. D. Brown. 1965. Control of aromatic biosynthesis: the multiplicity of 7-phospho-2-oxo-3 deoxy-D-arabino-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating) in *E. coli* W. Biochim. Biophys. Acta **104**:377–389.
13. Gòrisch, H., and F. Lingens. 197. 3-deoxy-D-arabino-heptulosonate-phosphate synthase of *Streptomyces aureofaciens* TU 24. I. Partial purification and properties. Biochim. Biophys. Acta **242**:617–629.

14. Jensen, R. A., and D. S. Nasser. 1968. Comparative regulation of isoenzymic 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetases in microorganisms. J. Bacteriol. **95**:188–196.

15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

16. Moldovanyi, I. Sz., and G. Dénes. 1968. Mechanism of the action and of the allosteric inhibition of DAHP synthase (tyrosine-sensitive) of *E. coli* W. Acta Biochim. Biophys. Acad. Sci. Hung. **3**:259–273.

17. Monod, J., G. H. Cohen-Bazire, and M. Cohen. 1951. Sur la biosynthèse de la β-galactosidase (lactase) chez *Escherichia coli*. La specificité de l'induction. Biochim. Biophys. Acta **7**:585–599.

18. Nicholls, P. W., and D. E.Rivett. 1972. The determination of tryptophan oxidation products using an amino acid analyser. J. Chromatogr. **65**:565–567.

19. Pittard, J., J. Camakaris, and B. J. Wallace. 1969. Inhibition of 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthetase (trp) in *Escherichia coli*. J. Bacteriol **97**:1242–1247.

20. Pittard, J., and S. Morrison. 1973. The purification and properties of DAHP synthetase (tyr) from *E. coli* K-12. Proc. Aust. Biochem. Soc. **6**:8.

21. Smith, L. C., J. M. Ravel, S. R. Lax, and W. Shive. 1962. The control of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthesis by phenylalanine and tyrosine. J. Biol. Chem. **237**:3566–3570.

22. Srinivasan, P. R., and D. B. Sprinson. 1959. 2-Keto-3-deoxy-D-arabo-hepatonic acid 7-phosphate synthetase. J. Biol. Chem. **234**:716–722.

23. Staub, M., and G. Dénes. 1969. Purification and properties of DAHP synthase (phenylalanine sensitive) of *Escherichia coli* K-12. II. Inhibition of activity of the enzyme with phenylalanine and functional group-specific reagents. Biochim. Biophys. Acta **178**:599–608.

24. Wallace, B. J., and J. Pittard. 1967. Genetic and biochemical analysis of the isoenzymes concerned in the first reaction of aromatic biosynthesis in *Escherichia coli*. J. Bacteriol. **93**:237–244.

25. Wallace, B. J., and J. Pittard. 1967. Chromatography of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate synthetase (trp) on diethylaminoethyl cellulose: a correction. J. Bacteriol. **9**:1279–1280.